PATENT
Attorney Docket No.: 022101-000320US
Client Ref. No.: 18915-US2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

David H. Gelfand et al.

Application No.: 10/052,417

Filed: January 17, 2002

For: THERMOSTABLE DNA POLYMERASES INCORPORATING NUCLEOSIDE TRIPHOSPHATES LABELED WITH FLUORESCEIN DYES

Customer No.: 41504

Confirmation No. 4095

Examiner: Jehanne Souaya Sitton

Technology Center/Art Unit: 1634

DECLARATION UNDER 37 CFR 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Nancy Jeneane Schoenbrunner, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2. I received my Ph.D. from Biozentrum der Universität Basel in January, 1997 and an Diplom Biochemie from Universität Bayreuth in December, 1992. I am an employee of Roche Molecular Systems, Inc. ("RMS") and I have been employed by RMS for 10 years. My current position is Research Leader. My work has involved characterization and design of DNA polymerases with novel properties for PCR applications. I develop and carry out

polymerase activity assays, assess substrate preference, enzyme kinetics and perform molecular modeling of the structures of natural and variant polymerases with various substrates. A copy of my CV is attached as Exhibit A.

3. I have reviewed the above-referenced patent application and I understand that the pending claims relate in part to polymerases having a mutation (corresponding to the E681R mutation in *Taq* polymerase, where "R" refers to arginine) that greatly reduces discrimination against fluorescein-labeled nucleotides. I have reviewed the Office Action mailed on April 25, 2008, and I understand that the patent Examiner has rejected the pending claims as obvious in view of any of three Brandis patent publications (US Patent No. 6,256,193; US Patent Publication 2002/0164591; or US Patent Publication 2006/0088879) (referred to jointly here as "Brandis") in view of Baker (US Patent No. 5,571,706) and Cormier (US Patent No. 5,418,155).

4. According to the April 25, 2008 Office Action, Brandis suggested that substitution with R would improve (reduce) discrimination properties of the mutant polymerase and that the improvement was predictable based on the teachings of Brandis and Baker (Office Action, page 11). The Office Action noted that Brandis provided data of mutations of "all amino acids in the same family as ... R" (Office Action, page 11). Further, the Office Action quoted Baker as stating: "It is well known in the biological arts that conservative amino acid substitutions can be made in protein sequences without affecting the function of the protein" (Office Action, page 3 and again on page 4). The Office Action concluded that:

(a) an "ordinary artisan would have been motivated to make the additional amino acid mutants... R;" (Office Action page 4)

(b) it would have been "obvious to try;" (Office Action page 4) and

(c) the "results would have been predictable" (Office Action page 4).

5. The Office Action indicated that the results presented in the Declaration of Dr. Gelfand (filed 1/3/2003), showing that R resulted in the best reduced discrimination, was <u>not</u>

a surprise because Dr. Gelfand used an assay involving different nucleotides and labels than used in the assay described in Brandis (Office Action, page 11). The Office Action indicated that Brandis predicted that the "degree of discrimination" would vary depending on the "base, dye, or linker" used (Office Action, page 11). The Office Action further stated that the Gelfand results were not a surprise because the level of discrimination for R "was on the same order of magnitude as a number of the same mutants which Brandis teaches" (Office Action, page 12).

6. I disagree with the logic and conclusions of the Office Action. Figure 1 of Dr. Gelfand's Declaration is the only data on the record that shows the effect of all possible 19 amino acid substitutions at the position of interest. Figure 1 of Dr. Gelfand's Declaration is therefore the best source of information on the record to directly compare the effect of different amino acid substitutions. This Figure shows the E683R mutation (corresponding to the E681 position in *Taq* polymerase) provided the best reduced discrimination for a fluorescein (HEX)-labeled dCTP. Specifically, the ratio of amount of fluorescein-labeled dCTP to unlabeled-dCTP required to reduce radioactively-labeled dCMP incorporation by 50% was 0.04. Data for E683H, E683K, and E683M are also provided in Figure 1 of Dr. Gelfand's Declaration and are summarized for convenience as follows:

Mutation	**Figure 1 ratio**	**Fold-improvement of R over other substitution**
E683R	0.04	
E683M	0.79	19.75
E683K	0.41	10.25
E683H	1.45	36.25
E683	19.05	476.25

Thus, Dr. Gelfand's data shows that R was almost 20 times better than M, and about 10 and 30-fold better than K and H, respectively.

7. In contrast, data presented by Brandis (e.g., in Table 2 of US Patent No. 6,265,193) indicates that M is the best mutation (showing least discrimination), with H and K ranking somewhere in the middle of the 16 different substitutions presented in Brandis. According to the Office Action (citing Baker): "It is well known in the biological arts that conservative amino acid substitutions can be made in protein sequences without affecting the function of the protein" (Office Action, page 3 and again on page 4). If R, H, and K reflected conservative amino acid changes, and one expected similar results for these substitutions, one would have expected that R would result in levels of discrimination similar to those observed for H and K (i.e., showing a 6-7 –fold improvement). If this was the expectation, it is not clear why one would make the R substitution at all since the H and K substitutions were not particularly good compared to a number of other substitutions. For example, M was 6.71 times "better" than H and 7.83 times "better" than K. Thus, one would have selected M and not proceeded with a substitution expected to work only as well as H or K.

8. Further, if R, H, and K reflected conservative amino acid changes, and one expected similar results for these substitutions, one would not have expected that R would show a 10-30-fold improvement relative to K or H (as observed by Gelfand) and one would certainly not have expected that the *ranking* of substitutions would change to such a great degree that R would be nearly 20-fold better than M. Indeed, if one of ordinary skill in the art expected conservative amino acid changes to have approximately the same effect, and then read the Brandis data, one would have been surprised to find that R was far superior than M as a substitution.

9. The Office Action appears to argue that one of ordinary skill in the art would not be surprised to find that different amino acid substitutions have superior discrimination depending on what type of labeled nucleotide was used in the particular assay involved in the determination. Specifically, the Office Action argued that "the ordinary artisan would have expected the exact levels of discrimination to differ based on the base, dye or linker used in the assay" (Office Action, sentence spanning pages 11-12). In support of this statement, the Office Action cited Brandis (col. 6, line 27-37) as stating that, "The precise degree of

discrimination will also vary in accordance with the specific fluorescently labeled nucleotide assayed, e.g., variations in base, dye, or linker. Mutant DNA polymerase of the invention may exhibit anywhere from a slight reduction in discrimination … to complete elimination of discrimination." The Office Action argued that "the type of nucleotide (dCTP vs ddCTP) as well as the label (Tet(II) vs HEX-2-PA used in Brandis and Dr. Gelfand's declaration are different" (Office Action, page 11).

10. While the exact nucleotide and label are different between the Brandis and Gelfand assays, they are not *very* different. As noted in the Office Action, Brandis uses ddCTP, whereas Gelfand uses dCTP. The additional 3' deoxy in ddCTP affects further incorporation of nucleotides to the ddCTP but the portions of a polymerase that interact with the 3' part of a labeled nucleotide are different from those that interact with the label part of a fluoroscein-labeled nucleotide. The 3' deoxy position is on the sugar of the nucleotide and very far away from the dye label which is attached to the base. Very different amino acids in the polymerase interrogate the base and moieties attached to the base compared to amino acids that interrogate the sugar. I am not aware of any evidence to date that indicates position 681 in *Taq*polymerase mediates discrimination against 3' deoxy nucleotides. Indeed, discrimination against 3' deoxy nucleotides is mediated by the nature of the amino acid at position 667 in *Taq* DNA polymerase near the active site of the enzyme, not at position 681. (Tabor, S., and Richardson, C. C. (1995) A single residue in DNA polymerases of the Escherichia coli DNA polymerase I family is critical for distinguishing between deoxy- and dideoxyribonucleotides, *Proc Natl Acad Sci U S A 92*, 6339-6343). Thus, one of skill in the art would not expect that presence or absence of a 3' hydroxyl (the difference between the nucleotides in Brandis and Gelfand) would affect discrimination against incorporation of fluorscein-labeled nucleotides.

11. The labels used in the Gelfand and Brandis assays are different, but the Tet(II) and HEX labels used by Brandis and Gelfand, respectively, are relatively similar (see attached Figure 1). Both dyes are members of the fluorescein family, the HEX label being substituted with six chloro moieties whereas the TET label has four chloro substitutions. While one would not expect to be able to directly compare quantities between two different assays measuring discrimination, one would expect that the *ranking* of amino acid substitutions would

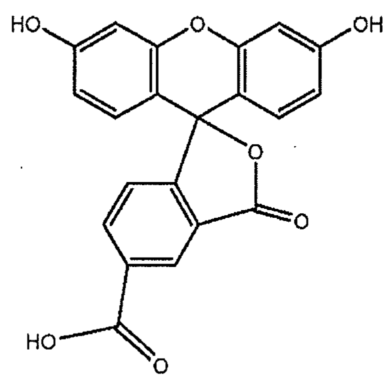
be similar when the assays use similar labeled nucleotides as is the case here. For example, if one assay showed that M was best and that H and K provided middle-of-the-pack results, one of ordinary skill in the art would generally expect a second assay for measuring similarly-labeled nucleotide discrimination to yield essentially the same ranking of substitutions, i.e., M better than H or K. In view of the similarity between the HEX and TET labels, I would not have expected the relative discrimination between HEX and TET-labelled nucleotides to be significantly different between different amino acid substitutions at position 681. Therefore, it is my opinion that one of ordinary skill in the art would have been surprised to learn that substitution with R results in such a superior level of reduced discrimination compared to other basic amino acid substitutions such as H and K and results in the best substitution overall, having a nearly 20-fold improvement over M.

12. In view of the above arguments, I do not believe that the reduced discrimination observed by Gelfand for the R substitution was predictable or expected. If one of skill would have expected similar activity from conservative amino acid changes, one would have expected an R substitution to generate "middle-of-the pack" results similar to the H or K substitutions, not activities that were nearly 10-fold better than the next-best substitution and nearly 20-fold better than M, which Brandis identified as best. Accordingly, it is my opinion that the effect of the R substitution on labeled nucleotide discrimination was not expected or obvious.

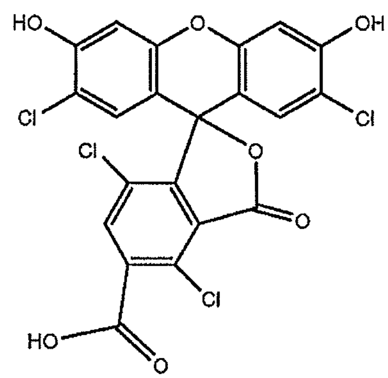
Dated: 1/20/2009

Nancy Jeneane Schoenbrunner

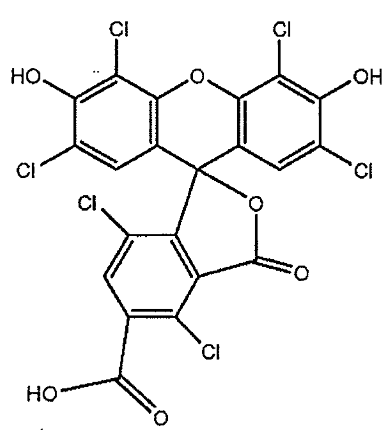
Figure 1



FAM



TET



HEX

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Employment

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> **1/05-PRESENT** RESEARCH LEADER, PROGRAM IN CORE RESEARCH,
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> **7/97-9/98** CONSULTANT, PROGRAM IN CORE RESEARCH.
>
> **SUMMARY:** STRUCTURE-BASED DESIGN OF THERMOSTABLE DNA POLYMERASES WITH NOVEL PROPERTIES; DEVELOPMENT OF ASSAYS FOR THE CHARACTERIZATION OF DESIGNER DNA POLYMERASES AND OTHER DNA ENZYMES; PCR APPLICATIONS OF DESIGNER DNA POLYERASES AND NUCLEIC ACID MODIFICATIONS; ASSIST OTHERS IN THE COMPANY (PUBLIC RELATIONS, TRAINING) BY GENERATING GRAPHICAL IMAGES FOR VARIOUS PURPOSES. DURING THESE PROJECTS I DIRECTED A SENIOR SCIENTIST TO CARRY OUT THE CLONING, EXPRESSION, PURIFICATION AND CHARACTERIZATION OF DESIGNED ENZYMES.

Expertise

- Nucleic Acid Thermodynamic Stability and Structure Prediction Software
- Computer-Aided Molecular Design of Proteins
- Bioinformatics and sequence analysis (GCG package, XSAE, and other Various Databases and Software)
- Programs for Molecular Graphics, Modeling and Dynamics (Moloc, WebLab Viewer, Xplor, Insight II)
- Measurement & computer analysis of complex protein folding and enzyme kinetics
- Development of Enzyme Assays
- Spectroscopic techniques (CD, Fluorescence, UV/VIS)
- Stopped-flow and quench flow techniques
- Limited expertise in NMR
- Fragmentation Analysis of Nucleic Acids using ABI 373, 377 and 3100
- Folding, Stabilization and Chemical Modification of recombinant proteins by various methods
- Programming and operation of Liquid Handling Stations

Educational Background

Biozentrum der Universität Basel *1993 - 1997*
Basel, Switzerland

> GRADUATE STUDIES IN THE DEPT. OF BIOPHYSICAL CHEMISTRY UNDER THE SUPERVISION OF DR. THOMAS KIEFHABER. DISSERTATION TOPIC: *"KINETIC AND EQUILIBRIUM STUDIES ON THE FOLDING MECHANISM OF TENDAMISTAT."*

PH. D. SUMMA CUM LAUDE

Universität Bayreuth *1988 - 1992*

Bayreuth, Germany

DIPLOMA PROGRAM IN BIOCHEMISTRY.
DIPLOM EXAM IN ORGANIC CHEMISTRY, BIOCHEMSTRY, GENETICS AND PLANT PHYSIOLOGY
DIPLOMA THESIS UNDER THE SUPERVISION OF PROF. DR. PAUL ROESCH. TOPIC: *"STRUCTURE PREDICTIONS OF MUTANTS OF P21RAS AND RIBONUCLEASE T1 USING MOLECULAR DYNAMICS."*
DIPLOMA "WITH AUSZEICHNUNG"; GRADE 1.2 (ON A SCALE OF 1-6 WITH 1 BEST)

Edinburgh University *1986- 1987*

Edinburgh, Scotland

JUNIOR YEAR ABROAD; COURSES IN MOLECULAR BIOLOGY, BIOCHEMSTRY(FIRST CLASS MERIT) AND PHYSICS (SECOND CLASS MERIT)

Georgetown University *1984 - 1988*

Washington, D.C.

MAJOR BIOLOGY / MINOR CHEMISTRY. BACHELOR OF SCIENCE *magna cum laude*.

University of California, Berkeley *1986*

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SUMMER COURSES IN HUMAN GENETICS AND MATHEMATICS

Languages

ENGLISH (NATIVE LANGUAGE)
GERMAN (FLUENT)

Awards received

FULBRIGHT SCHOLARSHIP 1988-1989 FOR STUDIES IN BIOCHEMISTRY AT THE UNIVERITY OF BAYREUTH, GERMANY
BIOLOGY MEDAL UPON GRADUATION FROM GEORGETOWN UNIVERSITY

Patents

N. SCHÖNBRUNNER, T. MYERS AND D. GELFAND. THERMOSTABLE OR THERMOACTIVE DNA POLYMERASE MOLECULES WITH ATTENUATED 3'-5' EXONUCLEASE ACTIVITY. U.S. SERIAL NO. 10/401,403, FILED ON APRIL 3, 2002.

E. SMITH, C. ELFSTROM, D. GELFAND, R. HIGUCHI, T. MYERS, N. SCHÖNBRUNNER, AND A. WANG. HIGH TEMPERATURE REVERSE TRANSCRIPTION USING MUTANT DNA POLYMERASES, U.S. SERIAL NO. 60/198,336, FILED ON APRIL 18, 2000.

Participation in Scientific Meetings

NUCLEIC ACID-BASED TECHNOLOGIES, JUNE 25-27, 2007. BALTIMORE, MD

KEYSTONE SYMPOSIUM ON STRUCTURAL GENOMICS JANUARY 29-FEBRUARY 3, 2006 KEYSTONE, CO

POSTER PRESENTATION AT ALL ANNUAL INTERNAL R&D FAIRS (2001-2003).

4TH ANNUAL HIV ANTI-VIRAL DRUG RESISTANCE SYMPOSIUM SPONSORED BY THE NATIONAL CANCER INSTITUE, DECEMBER 7-10, 2003, CHANTILLY, VA.

ANNUAL MEETING OF THE AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, APRIL 12-15, 2003 SAN DIEGO, CA WITH POSTER PRESENTATION.

GORDON RESEARCH CONFERENCE ON NUCLEIC ACIDS, BRISTOL, RI, JUNE 2-7, 2002 WITH POSTER PRESENTATION.

KEYSTONE SYMPOSIUM ON MOLECULAR MECHANISMS IN DNA REPLICATION AND RECOMBINATION, TAOS, NEW MEXICO 2/16-2/22/1999 WITH POSTER PRESENTATION.

23RD MEETING OF THE FEDERATION OF EUROPEAN BIOCHEMICAL SOCIETIES, BASEL, SWITZERLAND 8/13-8/18/1995 WITH POSTER PRESENTATION *"TFE-Induced Structure of the all β-sheet Protein Tendamistat"*

EUROPEAN RESEARCH CONFERENCE ON PROTEIN FOLDING AND STABILITY, SAN FELIU DE GUIXOLS, SPAIN 4/8-4/13/1995 WITH POSTER PRESENTATION *"Equilibrium Intermediates of the all β-sheet Protein Tendamistat"*

SUMMER SCHOOL "UNDERSTANDING PROTEIN MOTIONS", KAROLINSKA INSTITUTE, STOCKHOLM, SWEEDEN 8/28-9/1/1994 WITH POSTER PRESENTATION *"Equilibrium Intermediate of the all β-Sheet Protein Tendamistat."*

Publications

OLGA BÜDKER, ELLEN FISS, DAVID GELFAND, TOM MYERS, RACHEL SHAHINIAN, CHRISTOPHER SIGUA & NANCY SCHÖNBRUNNER, *STRUCTURE-BASED DESIGN AND CHARACTERIZATION OF CHIMERIC THERMOPHILIC DNA POLYMERASES WITH ATTENUATED 3'-5' EXONUCLEASE ACTIVITY: APPLICATION TO LONG RT-PCR.* Biochemistry 45(42): 12786-95.

NANCY SCHÖNBRUNNER & THOMAS KIEFHABER.
Folding of the all β-sheet Protein Tendamistat: Two-state Folding without Molecular Collapse. J. MOL. BIOL. (1997) **268** 526-538.

NANCY SCHÖNBRUNNER, MATHIAS SCHARF & THOMAS KIEFHABER.
Probing the Rate-Limiting Steps in Tendaistat Folding by Disulfide Replacement. BIOCHEMISTRY (1997) **36** 9057-9065.

NANCY SCHÖNBRUNNER, JOSEF WEY, JOACHIM ENGELS, HOLGER GEORG & THOMAS KIEFHABER.
Native-like β Structure in a Trifluorethanol-induced Partially Folded State of the all β-sheet Protein Tendamistat. J. MOL. BIOL. (1996) **260** 432-445.

Posters

DAVID E. BIRCH, ELLEN H. FISS, DAVID H. GELFAND, NANCY J. Schönbrunner, Rachel M. Shahinian, Sherry Zhang, and Thomas W. Myers. UTILIZATION OF A NOVEL DESIGNER DNA POLYMERASE FOR RT/PCR OF HIV-1 RNA IN HIV DRUG RESISTANCE GENOTYPING ASSAYS. 3RD ANNUAL RMD R&D FAIR NOVEMBER 12, 2003 ALAMEDA, CA.

OLGA BUDKER, NANCY SCHOENBRUNNER, ELLEN FISS, RACHEL SHAHINIAN, DAVID GELFAND AND TOM MYERS. DESIGNER DNA POLYMERASES WITH ATTENUATED PROOFREADING ACTIVITY FOR LONG RT-PCR. ANNUAL MEETING OF THE AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, APRIL 12, 2003 SAN DIEGO, CA

RM SHAHINIAN, DE BIRCH, EH FISS, DH GELFAND, NJ Schönbrunner, and TW Myers. Going the Distance: Extending the Usefulness of RT/PCR. 2nd Annual RMD R & D Fair, November 6, 2002 Berkeley, CA.

NANCY SCHOENBRUNNER, OLGA BUDKER, ELLEN FISS, RACHEL SHAHINIAN, DAVID GELFAND AND TOM MYERS. DESIGNER DNA POLYMERASES WITH ATTENUATED PROOFREADING ACTIVITY FOR LONG RT-PCR. GORDON RESEARCH CONFERENCE ON NUCLEIC ACIDS, BRISTOL, RI, JUNE 2003.

NANCY Schönbrunner, Barrett Allen, Stephen Will, Thomas W. Myers, and David H. Gelfand. Kinetic Studies of Primer-Dimer Suppression by Major Groove Alkylation. 1st Annual RMD R & D Fair, October 2, 2001 Pleasanton, CA.

NANCY Schönbrunner, ELLEN FISS, OLGA BUDKER, THOMAS W. MYERS, AND DAVID H. GE.LFAND. THE DEVELOPMENT AND CHARACTERIZATION OF A FAMILY OF CHIMERIC DESIGNER DNA POLYMERASES WITH ATTENUATED 3'-5' EXONUCLEASE ACTIVITY FOR RT/PCR. 1st Annual RMD R & D Fair, October 2, 2001 Pleasanton, CA.

E. Smith, A. Wang, C. Sigua, N. Schönbrunner, T. Myers, D. Gelfand *HIGH TEMPERATURE MAGNESIUM-ACTIVATED REVERSE TRANSCRIPTION BY THERMOSTABLE DNA POLYMERASE*, SAN DIEGO CONFERENCE ON MOLECULAR TECHNOLGIES IN HEALTH CARE, NOV. 16-18, 2000.

R. Nersesian, D. Birch, V. Bodepudi, D. Fong, D. Gelfand, G. Hillman, N Schönbrunner, R. Shahinian, C. Sigua, T. Ryder, T. Webster, K. Wu, T. Myers
HIV-1 DRUG RESISTANCE/GENOTYPING BY A MICROARRAY BASED ASSAY, 4TH INTERNATIONAL WORKSHOP ON HIV DRUG RESISTANCE & TREATMENT STRATEGIES, JUNE 12-16, 2000.

T. Myers, D. Birch, V. Bodepudi, D. Fong, D. Gelfand, L. Kolmodin, R. Nersesian, R. Shahinian, C. Sigua, N. Schönbrunner, R. Resnick, K. Wu, T. Ryder
MICROARRY BASED HIV-1 DRUG RESISTANCE/GENOTYPING ASSAY, 2ND FRANKFURT SYMPOSIUM ON THE CLINICAL IMPLICATIONS OF HIV DRUG RESISTANCE, FEB. 25-27, 2000.

R. Nersesian, D. Fong, L Kolmodin, R. Shahinian, C. Sigua, N. Schönbrunner, R. Resnick, D. Birch, D. Gelfand, T. Myers
DEVELOPMENT OF A MICROARRAY BASED HIV-1 DRUG RESISTANCE/GENOTYPING ASSAY, 7TH CONFERENCE ON RETROVIRUSES AND OPPORTUNISTIC INFECTIONS, JAN. 30-FEB. 2, 2000.

References

UPON REQUEST